REMARKS

Claim amendments

Claims 2-9 and 11 have been canceled.

Claim 10 has been amended to depend from Claim 1.

Claims 12-15 have been added. Support for Claims 12 and 15 can be found, for example, in paragraph 0016 of the specification. Support for Claims 13 and 14 can be found, for example, in the working examples, particularly paragraphs 0045-0047, of the specification.

No new matter has been added.

PTO-892 Form

Applicants direct the Examiner's attention to a typographical error on the PTO-892 form that accompanied the Office Action. The PTO-892 form lists U.S. Patent No. 6,335,252 B1. However, Applicants believe that U.S. Patent No. 6,355,252 B1 should have been listed, since the claims in the subject application were rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-3 of U.S. Patent No. 6,355,252.

Rejection of Claims 1 and 7-11 under 35 U.S.C. §112, first paragraph

Claims 1 and 7-11 are rejected under 35 U.S.C. §112, first paragraph because the "specification does not set forth sufficient teachings to allow one skilled in the art to use the claimed medicament for treatment or prophylaxis of infectious diseases" (Office Action, page 3). Specifically, the Examiner states that the specification does not "provide teachings to establish effective dosages or methods of administration of the claimed recombinant poxvirus [to] treat infections" or "description or exemplification of how to use the medicament for prevention, diagnosis, alleviation, treatment, or cure of a disease in the animal to which the substance is administered" (Office Action, page 3).

Applicants respectfully disagree. "The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with

information known in the art without undue experimentation" (United States v. Teletronics, Inc. 8 U.S.P.Q.2d, 1217, 1223 (Fed. Cir. 1988).

In the specification as filed, Applicants provide working examples in which an effective dose of the claimed medicament was administered subcutaneously to a rabbit model, which as discussed below, is a standard model in the field. Furthermore, poxviruses have been used by those of skill in the art for hundreds of years, and thus, effective dosages and methods of administering poxviruses are well known in the art. It is well established that "a patent need not teach, and preferably omits, what is well known in the art" (Hybritech, Inc. v. Monoclonal Antibodies, Inc., 231 USPQ 81, 94 (Fed. Cir. 1986)).

The Examiner further states that "[p]redicting the therapeutic effect of a recombinant viral construct is uncertain and must be tested for each recombinant that may include deletions, insertions, or both, and cites the results in Jackson *et al.* as teaching the "critical importance of testing a vaccine in order to ensure that it actually performs with the expected result" (Office Action, pages 3-4).

Applicants respectfully disagree. Applicant's invention is directed to a poxvirus in which a virulence gene (A41L) has been deleted (one deleted gene). The A41L protein is a chemokine receptor which acts to reduce the host immune response. Using a rabbit model, Applicants demonstrate that deletion of the A41L gene provided the particular improvement of an enhanced immune response to the poxvirus (the vΔA41L poxvirus), compared to the immune response to the existing poxvirus vaccine that is well known in the art and included the A41L gene (the A41L poxvirus).

In contrast, Jackson *et al.* used a recombinant poxvirus containing an additional murine gene (IL-4). IL-4 as used in Jackson *et al.* was known to inhibit antiviral immune responses (Jackson *et al.*, p. 1206 lines 1-5), therefore it was not surprising that mousepox-IL-4 had enhanced virulence. However, Applicants fail to find support for the Examiner's assertion that Jackson *et al.* teach the "critical importance of testing a vaccine in order to ensure that it actually performs with the expected result". Applicants respectfully request clarification.

Nevertheless, Applicants have tested the claimed medicament in an acceptable animal model. Based on Applicants' *in vivo* data and the well established use of poxvirus vaccines in the art, one of skill in the art would reasonably expect that, as with existing poxvirus vaccines,

Applicants' claimed recombinant poxvirus could be used as a medicament for treatment or prophylaxis.

It is the Examiner's opinion that the specification "***speculates*** (see [0087]) that the removal of the A41L gene is likely to increase the immunogenicity and safety of VV strains such as VV MVA, and that the immunogenic VV strain can be used as [a] therapeutic vaccine against various cancers and a range of pathogens" and "has shown (see [0056]) that injecting a rabbit subcutaneously with a recombinant A41L deleted VV causes greater infiltration of the surrounding tissue by leukocytes, implying that the deletion make the virus more visible to the immune system" (Office Action, page 4, emphasis added). The Examiner further notes that "the VV strain tested is not a rabbit pathogen, therefore the observed effects cannot be extrapolated to the natural host" (Office Action, page 4).

Applicants respectfully disagree. The vaccinia virus is an attenuated strain of artificial origin, thought to have originally been taken from cows or horses. Vaccinia causes local, rather than systemic, infection in all strains of mammals and it is indeed this property that has caused it to be used as a smallpox vaccine. In the specification as filed, Applicants demonstrate that by deleting the A41L gene, a poxvirus of the claimed invention becomes more immunogenic, which has been accepted by the Examiner. It would not require undue experimentation to merely use this as, for example, a smallpox vaccine, as it is clearly at least equivalent to conventional vaccinia strains.

Applicants have provided an enabling disclosure for the claimed invention which is further evidenced by consideration of the Wands factors below.

Scope and breadth of the claims

The claims are directed to medicaments/vaccines comprising poxviruses in which the A41L gene has been deleted. In this respect the claimed invention is an improvement on pox virus vaccines such as vaccinia, or fowl pox vaccines for poultry. It is also contemplated that pox viruses lacking A41L could be of use in recombinant pox virus vaccines, such as those expressing malarial or other pathogens. These were part of the state of the art as of the filing date (*e.g.* U.S. Patent No. 4,722,848; Claims 3-7). The claims are therefore limited to a particular improvement (one deleted gene) on the state of the art.

Nature of the invention

As discussed above, the invention is an improvement on existing pox virus vaccines.

Relative level of skill in the art

The skill level in the art is high. Those skilled in the art would include molecular biologists and virologists, able to create knockout pox viruses by site specific recombination, and immunologists able to test the immunogenicity of the viruses in animals and humans. It would also be routine for these artisans to identify genes with 80% or more sequence homology to A41L in other poxviruses by using sequence databases.

State of the art and knowledge in the prior art

Poxviruses had been used as vaccines for hundreds of years. Recombinant poxviruses had been developed as vaccines at least 15 years before the priority date of the present invention (see US Patent No. 4,722,848 cited above).

Predictability in the art

As mentioned, recombinant and mutant poxviruses had been studied for over 15 years. Applicants fail to find the teaching in the Jackson *et al,* of unpredictability in the art; as discussed above Jackson *et al.* used mousepox expressing a murine immunosuppressant gene which, along the lines expected, enhanced virulence and mortality by suppressing the host immune response.

Amount of guidance provided by the inventors

In the application as filed, Applicants demonstrated vaccination with vaccinia virus lacking A41L in a rabbit model which is a standard model in this field, and showed enhanced immunogenicity. Applicants also teach that recombinant poxviruses expressing heterologous genes could have their immunogenicity improved by deletion of A41L.

Presence or absence of working examples

As discussed above, Applicants provide a working example of vaccination of rabbits, which is an accepted model in the field.

Quantity of experimentation to make and use the invention

As discussed above, the A41L-deleted vaccinia virus described in the specification could be formulated as is into a smallpox vaccine, which would involve no experimentation at all. It would be expected to be at least as effective as a conventional smallpox vaccine, but with enhanced immunogenicity.

Finally, Applicants direct the Examiner's attention to Clark *et al., J. Gen. Virol., 87:*29-38 (2006), a subsequent publication of one of the inventors, being filed concurrently as the Exhibit. As Applicants teach in the specification as filed, Clark *et al.* show that deletion of A41L from MVA improves vaccine efficiency *in vivo* (Clark *et al.*, pages 34-35).

Applicants have provided an enabling disclosure for the full scope of the claimed invention.

Rejection of Claims 1 and 7-11 under 35 U.S.C. §112, first paragraph

Claims 1 and 7-11 are rejected under 35 U.S.C. §112, first paragraph as failing to comply with the written description requirement. The Examiner states that the "claims encompass a poxvirus that do not express a large genus of polypeptides" and "[w]hile one of skill can determine whether the poxvirus fails to express SEQ ID NO: 2 (a native A41L protein), one cannot determine whether the poxvirus fails to express variants of SEQ ID NO: 2" (Office Action, page 6). The Examiner further states that the "variants of SEQ ID NO: 2 can include any sort of modification . . ., any number of modification, at any point along the sequence set forth in SEQ ID NO: 2" and "[s]ince Applicant has not provided a representative number of species of poxviruses that fail to express proteins that have less than 100% sequence identity to SEQ ID NO: 2, or a core region of SEQ ID NO: 2 that is retained in the variant such that one would know that a variant A41L protein has not been expressed in the poxvirus, one would not be put in possession of the large genus claimed" (Office Action, page 6).

Applicants respectfully disagree. Written description can be met by "show[ing] that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics . . *i.e.,* complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics" (Guidelines for Examination of Patent Applications under 35 U.S.C. §112, first paragraph, "Written Description" Requirement, 66 Fed. Reg. 1099, 1106 (Jan. 5, 2001)).

Applicants' claimed invention is directed to a medicament comprising a recombinant poxvirus which is genetically engineered to be incapable of expressing a native A41L protein, together with a pharmaceutical acceptable carrier for use in treatment or prophylaxis. In the specification as filed, Applicants describe construction of a VV strain WR mutant lacking the A41L gene using a known method (Falkner and Moss,1990). In addition to the VV WR A41L gene, Applicants describe other poxviruses which express a native A41L protein, and cite references providing the sequences of these other known A41L genes in the specification as filed. Also known to those of skill in the art at the time of Applicant's invention was that these known A41L proteins shared significant amino acid sequence identity.

The Examiner's attention is directed to Applicants' teaching that the "DNA sequence data on orthopoxvirus genomes indicated that the A41L gene is highly conserved" (specification, paragraph [0073]). Specifically, Applicants teach that "[i]n all three strains of variola major virus that have been sequenced there is an open reading frame (ORF) with more than 95% ***amino acid identity*** to the VV WR A41L protein termed 16L in strain Harvey (Aguado *et al.*, 1992), A44L in strain Bagladesh-1975 (Massung *et al.*, 1994) and A46L in strain India-1967 (Shcheikunov *et al.*, 1994)" (specification, paragraph [0008], page 4, emphasis added). In addition, Applicants note that "gene A41L encodes a 30 kDa protein that is secreted from cells infected by all strains of orthopox examined including 16 strains of VV and 2 strains of cowpox virus. In addition, it is predicted to be expressed from all 3 strains of variola major virus for which sequence data is available" (specification, paragraph [0010], page 5; see also paragraph [0062], page 16 and paragraph [0064], page 17).

Citing the *Fiers v. Revel* and *Amgen Inc v. Chugai Pharmaceutical* cases, the Examiner states that "[a]dequate written description requires more than a mere statement that it is part of

the invention and reference to a potential method of isolating it" . . . the "compound itself is required" (Office Action, page 7). However, as discussed below, the *Fiers* and *Amgen* cases are not on point here. Rather, Applicants direct the Examiner's attention to *Invitrogen Corp. v. Clontech Laboratories Inc* 77 U.S.P.Q.2d 1161 (CAFC 2005) which is directly applicable to the facts of the subject application.

In *Fiers*, Appellant's specification lacked written description for the claimed DNA encoding an IFNβ polypeptide because only a method for isolating a fragment of the DNA and a method for isolating the mRNA coding for IFNβ was disclosed in the specification. In *Amgen*, Appellant's specification lacked written description for the claimed sequences encoding functional analogs of human EPO because only the DNA sequence of human EPO was disclosed in the specification.

In *Invitrogen*, Appellant's specification provided adequate written description for an isolated polypeptide having DNA polymerase activity and substantially reduced RNase H activity encoded by a modified reverse transcriptase (RT) nucleotide sequence. Thus, "Invitrogen thereby claims a compound (the polypeptide or genetically engineered RT) in terms of biological functions", rather than its particular sequence (*Invitrogen* at 1174). In the specification as filed, Appellant provided a working example of mutating a known gene that encoded a naturally occurring RT (MMLV) which resulted in a mutant with the claimed properties; taught that the invention could be applied to other RT genes from other retroviruses (HTLV-1, BLV, RSV, HIV); and provided the references describing the sequences of these other RT genes which were known in the art at the time of filing. Based on the facts, the court found that the specification provided adequate written description for the claimed invention because:

> in addition to the sequence recited in the specification at bar, "at the time of the invention, the sequences of other RT genes were known and members of the RT gene family shared significant homologies from one species of RT to another." The written description teaches that the invention can be applied to RT genes of other retroviruses including HTLV-1, BLV, RSV, and HIV . . . . the specification cites references providing the known nucleotide sequences of these RT genes . . . (*Invitrogen* at 1185).

Thus, as in the *Invitrogen* case, Applicants provide a specific example of the VV WR genetically engineered to be incapable of expressing the native A41L gene in the subject application. Furthermore, at the time of the invention, the sequences of other A41L genes were known, these members of the A41L gene family shared sequence identity, and in the specification as filed, Applicants teach that the invention can be applied to these other A41L genes and cite references which provide the A41L sequences.

Applicants have provided written description for the claimed invention.

Rejection of Claims 1 and 7-11 on the ground of nonstatutory obviousness-type double patenting

Claims 1 and 7-11 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-3 of U.S. Patent No. 6,355,252.

Once the claims of the present application are deemed otherwise allowable, and should those claims cover subject matter that is not patentably distinct from that covered by the claims of the 6,355,252 patent, Applicant will submit an appropriate terminal disclaimer by the common Assignee of the present application and the 6,355,252 patent. A terminal disclaimer is not an admission or comment regarding the merits of the rejection (*Quad Envtl. Techs. Corp. v. Union Sanitary Dist.* , 946 F.2d 870 (Fed. Cir. 1991)).

CONCLUSION

In view of the above amendments and remarks, it is believed that all claims are in condition for allowance, and it is respectfully requested that the application be passed to issue. If the Examiner feels that a telephone conference would expedite prosecution of this case, the Examiner is invited to call the undersigned.

Respectfully submitted,

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Deletion of gene *A41L* enhances vaccinia virus immunogenicity and vaccine efficacy

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Vaccinia virus (VACV) is the vaccine that was used to eradicate smallpox and is being developed as a recombinant vaccine for other pathogens. Removal of genes encoding immunomodulatory proteins expressed by VACV may enhance virus immunogenicity and improve its potential as a vaccine. Protein A41 is a candidate for removal, having sequence similarity to the VACV chemokine-binding protein, vCKBP, and an association with reduced inflammation during dermal infection. Here, it is shown that, at low doses, VACV strain Western Reserve (WR) lacking *A41L* (vΔA41L) was slightly more virulent than wild-type and revertant controls after intranasal infection of BALB/c mice. The primary immune response to vΔA41L was marked by an increase in the percentage of VACV-specific gamma interferon-producing $CD8^+$ T cells and enhancement of cytotoxic T-cell responses in the spleen. However, this augmentation of cellular response was not seen in lung infiltrates. Splenic $CD8^+$ T-cell responses were also enhanced when VACV strain modified vaccinia virus Ankara (MVA) lacking *A41L* was used to immunize mice. Lastly, immunization with VACV MVA lacking *A41L* provided better protection than control viruses to subsequent challenge with a 300 LD_{50} dose of VACV WR. This study provides insight into the immunomodulatory role of A41 and suggests that MVA lacking A41 may represent a more efficacious vaccine.

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INTRODUCTION

Vaccinia virus (VACV) is a large, double-stranded DNA virus that replicates in the cytoplasm and has roughly 200 genes (Goebel *et al.*, 1990). Approximately one-half of the encoded proteins are non-essential for virus replication and are involved in virus–host interactions such as immune evasion (Moss, 2001). VACV is the prototypic orthopoxvirus, the vaccine used to eradicate smallpox (Fenner *et al.*, 1988) and a vector for development of vaccines for other diseases (Panicali *et al.*, 1983; Smith *et al.*, 1983). Recently, limited vaccination against smallpox was reintroduced because of fears of bioterrorism. However, the live vaccines used during the smallpox-eradication campaign had a relatively poor safety record (Lane *et al.*, 1969) and a safer smallpox vaccine is needed. One approach is to use the highly attenuated VACV strain modified vaccinia virus Ankara (MVA) (Staib *et al.*, 2004). MVA was obtained by extensive passage of VACV Ankara in chicken embryo fibroblasts (CEFs) (Mayr & Danner, 1978) and, during passage, lost DNA from several regions of the genome (Meyer *et al.*, 1991), including some genes encoding immunomodulators (Antoine *et al.*, 1998; Blanchard *et al.*, 1998). In addition, MVA lost the ability to replicate in most mammalian cells (Sutter & Moss, 1992; Carroll & Moss, 1997). MVA was used towards the end of the smallpox-eradication campaign in Germany without complication (Stickl *et al.*, 1974) and is avirulent even in immunosuppressed animals (Stittelaar *et al.*, 2001; Wyatt *et al.*, 2004). Nonetheless, immunization with MVA induced robust $CD8^+$ T-cell responses comparable to those induced by more virulent VACV strains, such as Western Reserve (WR), Dryvax or Lister, while also providing protection in animal-challenge models (Belyakov *et al.*, 2003; Earl *et al.*, 2004; Wyatt *et al.*, 2004; Stittelaar *et al.*, 2005).

$CD8^+$ T cells have been reported to play a role in protection against disease induced by poxviruses (Belyakov *et al.*, 2003; Drexler *et al.*, 2003; Snyder *et al.*, 2004; Tscharke *et al.*, 2005) and other pathogens, such as *Mycobacterium tuberculosis*, human immunodeficiency virus (HIV) and malaria (Kaech *et al.*, 2002; Kaufmann & McMichael, 2005). With this in

†These authors contributed equally to [illegible]

EXHIBIT

mind, vaccination strategies utilizing MVA to generate strong CD8$^+$ T-cell responses are being developed (Sutter & Moss, 1995). Preliminary data suggest that DNA prime–MVA boost is an effective way of generating a strong CD8$^+$ T-cell response (Hanke *et al.*, 1998; Schneider *et al.*, 1998) with high-avidity CD8$^+$ T cells (Estcourt *et al.*, 2002).

Although MVA is already a useful vector, its immunogenicity may be enhanced by the removal of other genes encoding immunomodulatory proteins that remain in the genome. MVA gene *184* encodes an interleukin 1β (IL-1β)-binding protein that is secreted from infected cells (Antoine *et al.*, 1998; Blanchard *et al.*, 1998), analogous to the soluble IL-1β receptor (IL-1βR) encoded by other VACV strains and cowpox virus (Alcamí & Smith, 1992, 1996; Spriggs *et al.*, 1992). Recently, it was shown that removal of this gene enhanced the CD8$^+$ T-cell response generated against MVA (Staib *et al.*, 2005). Another immunomodulatory protein expressed by MVA is A41.

Gene *A41L* was studied previously in VACV strain WR and is conserved in all 16 strains of VACV tested (Ng *et al.*, 2001). A41 has amino acid similarity to a family of poxvirus proteins that are secreted from the infected cell and bind CC chemokines or other ligands. These include the T1 protein from Shope fibroma virus and a 35 kDa protein from several VACV strains that each bind CC chemokines (Graham *et al.*, 1997; Smith *et al.*, 1997; Alcamí *et al.*, 1998) and a related protein from *Orf virus*, celled GIF, that binds IL-2 and granulocyte–macrophage colony-stimulating factor (Deane *et al.*, 2000). The ligand for A41 remains unknown, but deletion of the gene from VACV strain WR suggested an immunomodulatory role for this protein. In a mouse intradermal model of infection (Tscharke & Smith, 1999, 2002), the mutant lacking the *A41L* gene induced larger lesions with a greater influx of inflammatory cells compared with control viruses, and the rate of virus clearance was accelerated (Ng *et al.*, 2001).

In this study, we have examined the immunogenicity of VACV strains WR and MVA lacking gene *A41L*. By using VACV strain WR vΔA41L, we show that the primary immune response following intranasal infection exhibits an increase in the relative number of virus-specific splenic CD8$^+$ T cells that display cytolytic activity or produce gamma interferon (IFN-γ). A similar increase was seen in the CD8$^+$ T-cell memory response against MVA recombinants lacking *A41L* compared with controls. This increase in virus-specific memory CD8$^+$ T cells was accompanied by increased protection in mice challenged intranasally with VACV WR. Therefore, not only was a particular arm of the immune response affected by A41, but removal of this immunomodulator created a more potent vaccine.

METHODS

Cells and viruses. VACV strain WR was grown in BSC-1 cells in Dulbecco's modified Eagle's medium (DMEM) with 10 % heat-inactivated fetal bovine serum (FBS) and was titrated as described previously (Mackett *et al.*, 1985). VACV WR lacking gene *A41L* (vΔA41L) and wild-type (vA41L) and revertant (vΔA41L-rev) control viruses were constructed previously (Ng *et al.*, 2001). VACV strain MVA was grown in CEFs in DMEM with 10 % FBS and was titrated on CEFs. To enumerate foci of infection, cell monolayers were stained with rabbit polyclonal antibody to VACV followed by biotinylated donkey anti-rabbit IgG antibody (Amersham Biosciences) and streptavidin–alkaline phosphatase (Sigma).

Construction of VACV strain MVA *A41L* deletion and revertant viruses. To construct an MVA lacking gene *A41L*, oligonucleotide primers were used to amplify the regions flanking *A41L* from MVA genomic DNA, as was done for VACV WR (Ng *et al.*, 2001). These PCR-generated fragments were cloned into a pGEM-based plasmid, pK1L, which also contained the VACV *K1L* host-range gene (Tscharke & Smith, 2002), and were sequenced to confirm fidelity. The same primers were used because the primer-binding sites are identical in MVA and VACV WR. The resultant plasmid, pdelA41L, was transfected into cells infected with a recombinant MVA virus expressing the *Plasmodium berghei* circumsporozoite protein (Pbcsp) (Schneider *et al.*, 1998) and parental (MVA-A41L) and deletion mutant (MVA-ΔA41L) viruses were constructed by transient dominant selection (Falkner & Moss, 1990), using the *K1L* host-range gene as a selectable marker (Tscharke & Smith, 2002). Recombinant viruses expressing the *K1L* gene replicated on RK_{13} cells, whereas those lacking this gene did not, but would grow on CEFs. The genotype of individual plaque isolates was screened by PCR. A revertant virus in which *A41L* was reinserted into its natural locus in MVA-ΔA41L was constructed using plasmid pA41Lrev and was called MVA-A41L-rev. pA41Lrev was similar to pdelA41L, except that it was based on pBluescript rather than pGEM and the full-length MVA *A41L* gene was ligated into the plasmid, rather than the *A41L*-flanking regions.

Immunization strategies. For infection with VACV WR or derivative viruses, groups of female BALB/c mice (6–8 weeks old) were anaesthetized and infected intranasally with 5×10^3 p.f.u. virus in 20 µl PBS. Mice were weighed individually and monitored for signs of illness (Alcamí & Smith, 1992). Any mouse that had lost >30 % of its weight compared with the weight on day 0 was sacrificed. Virus present in the lung and spleen was released by homogenization, freeze–thawing three times and sonication and was titrated by plaque assay on BSC-1 cells. For immunizations with MVA or derivative viruses, mice were infected subcutaneously with 10^8 p.f.u. virus in 200 µl PBS. After 3 weeks, this procedure was repeated. Three weeks later, the animals were sacrificed and their spleens were processed for immunological analyses (see below). For challenge experiments, mice were immunized subcutaneously with either 10^6 or 10^7 p.f.u. MVA. Four weeks later, animals were infected intranasally with 3×10^6 p.f.u. VACV WR and their body weight and signs of illness were monitored. A sample of the inoculum used to infect each group of mice was titrated to ensure that the correct dosage had been administered.

Recovery of immune cells from infected mice. For VACV WR infections, mice were sacrificed and lavaged as described by Hussell *et al.* (1997). Bronchial alveolar lavage (BAL) samples were centrifuged at 800 *g* to obtain BAL cells. Leukocytes were obtained from lung homogenates by enzymic digestion, lysis of erythrocytes and centrifugation through 20 % Percoll (Sigma-Aldrich) as described by Lindell *et al.* (2001). Leukocytes were then resuspended in RPMI/5 % FBS. Splenocytes were obtained by homogenization of spleens, lysis of erythrocytes, centrifugation and resuspension in RPMI/5 % FBS. Live cells in all single-cell suspensions were enumerated using a haemocytometer and trypan blue exclusion.

Cell phenotyping, intracellular cytokine staining (ICS) and flow cytometry. Single-cell suspensions of BAL, lung or spleen cells

were blocked with 10 % normal rat serum and 0·5 μg Fc block [BD Biosciences (Pharmingen)] in fluorescence-activated cell sorting (FACS) buffer (PBS containing 0·1 % BSA and 0·1 % sodium azide) on ice for 30 min. Cells were stained with appropriate combinations of fluorescein isothiocyanate-, phycoerythrin (PE)- or tricolour-labelled anti-CD3, anti-CD8, anti-CD4, anti-F4/80 (macrophage), anti-Ly6G (neutrophil) or anti-pan NK (DX5) and the relevant isotype antibody controls (Pharmingen). The distribution of cell-surface markers was determined on a FACScan flow cytometer with CellQUEST software (BD Biosciences). A lymphocyte gate was used to select at least 20 000 events.

For ICS, single-cell suspensions of spleen or lung cells (10^6) were stimulated with specific peptides or VACV-infected cells or were mock-stimulated. For peptide stimulation, P815 cells were incubated with the relevant peptide at 10 μg ml^{-1} [Pb9 epitope, SYIPSAEKI; VACV-9 epitope, VGPSNSPTF (D. C. Tscharke, unpublished results)] for 2 h and then washed three times. Mock stimulations were P815 cells incubated with an irrelevant peptide (HIV-10 epitope, RGPGRAFVTI). For stimulation with VACV, P815 cells were infected with VACV WR at 10 p.f.u. per cell for 2 h in RPMI and then for a further 2 h after addition of 10 ml RPMI/10 % FBS. Cells were then washed three times. Effector and stimulator cells were incubated in RPMI/10 % FBS (ratio 5 : 1) for 5 h at 37 °C. After 90 min, brefeldin A (10 μg ml^{-1}; Sigma) was added. Cells were stained for cell-surface markers (as above) and then fixed with 2 % paraformaldehyde in PBS for 30 min at room temperature (RT). Cells were permeabilized with 0·5 % saponin in FACS buffer for 10 min. PE-conjugated anti-mouse IFN-γ or tumour necrosis factor alpha (TNF-α) (Pharmingen) was added for a further 30 min at RT and the cells were washed once with 0·5 % saponin in FACS buffer and twice in FACS buffer alone. Cells were analysed on a BD Biosciences flow cytometer, collecting data on at least 100 000 lymphocytes.

ELISPOT. The IFN-γ ELISPOT assay using peptide stimulation has been described by Miyahira *et al.* (1995). Briefly, assay plates (Millipore Multiscreen HA, MAHA54510) were coated overnight with 50 μl rat anti-mouse IFN-γ mAb (4 μg ml^{-1}; Pharmingen) at 4 °C. Plates were washed extensively with PBS and blocked for 2 h at RT with RPMI/10 % FBS. Splenocytes resuspended in RPMI/2·5 % FBS were added and diluted twofold to yield at least three concentrations. For experimental wells, 2 μg ml^{-1} (final concentration) of peptides Pb9 or VACV-9 or 50 000 WR-infected P815 cells were added to each well and the volume was adjusted to 100 μl with RPMI/2·5 % FBS. Samples were assayed in triplicate. Negative-control wells contained splenocytes and 50 000 uninfected P815 cells or an irrelevant peptide (HIV-10) at 2 μg ml^{-1}.

Plates were incubated at 37 °C for 12 h, washed with PBS and incubated for 2 h at RT with biotinylated rat anti-mouse IFN-γ mAb (4 μg ml^{-1}; Pharmingen). Plates were washed again with PBS and incubated with streptavidin–alkaline phosphatase (Sigma, diluted 1 : 1000) for 2 h at RT. Plates were washed with PBS again and developed with BCIP/NBT (Sigma). Spots were counted by using an AID ELISPOT reader with version 3.0 software (Autoimmun Diagnostika).

Cytotoxic T-lymphocyte (CTL) cytolysis assay. VACV-specific CTL activity in single-cell suspensions from lung and spleen was assayed by ^{51}Cr-release assay on VACV-infected P815 cells. P815 cells were mock-infected or were infected with VACV WR at 10 p.f.u. per cell for 2 h at 37 °C in 250 μl RPMI (−FBS) in the presence of $Na_2{}^{51}CrO_4$ (2 MBq in 1×10^6 cells). These cells were washed twice, left for 2 h in 10 ml RPMI/10 % FBS and washed again. Serial dilutions of effector cells were incubated in duplicate cultures with either mock-infected or VACV-infected target cells in 96-well V-bottomed plates. Cells were collected by centrifugation after 6 h and 50 μl supernatant was transferred to a Lumaplate-96 (Packard Instrument Company, Inc.) and counted. The percentage of specific ^{51}Cr release was calculated as specific lysis = [(experimental release − spontaneous release)/(total detergent release − spontaneous release)] × 100. The spontaneous-release values were always < 5 % of total lysis.

Statistical analysis. Student's *t*-test (two-tailed) was used to test the significance of the results ($P < 0·05$).

RESULTS

Deletion of *A41L* from VACV WR enhances virulence slightly in a murine intranasal-infection model

Previously, it was reported that deletion of the *A41L* gene did not affect the outcome of intranasal infection of BALB/c mice with doses of 10^4 p.f.u. or greater (Ng *et al.*, 2001). To investigate whether there were differences at lower doses of virus, mice were infected with 5×10^3 p.f.u. vA41L, vΔA41L or vA41L-rev and weight loss and signs of illness were monitored (Fig. 1a). Mice infected with vΔA41L lost more weight than those infected with either control virus ($P < 0·05$ on day 7) and also showed greater signs of illness

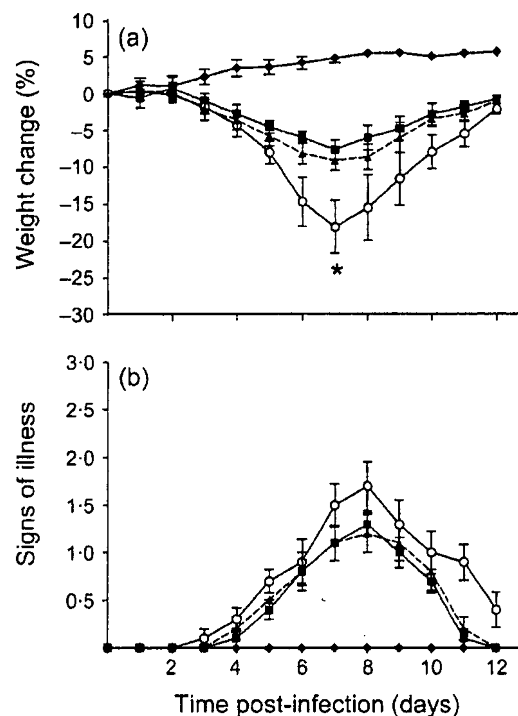

Fig. 1. Weight change of mice infected intranasally with VACV WR viruses. Groups of six BALB/c mice were mock-infected (◆) or infected with 5×10^3 p.f.u. vA41L (▲), vΔA41L (○) or vA41L-rev (■) and their weights (a) and signs of illness (b) were measured daily. Weights are compared with weight on day zero. Data are presented as the mean ± SEM. *P* values indicate a significant difference (*$P < 0·05$) between vΔA41L and both wild-type VACV and vA41L-rev at day 7.

(Fig. 1b), although the latter difference was not statistically significant. Similar results were obtained in a repeat experiment. To investigate whether this slightly enhanced virulence was due to increased virus replication, virus titres in lungs and spleen were measured at 3, 6 and 9 days post-infection (p.i.). This revealed no significant differences between groups (data not shown), indicating that the enhanced virulence of vΔA41L was not due to enhanced replication or spread.

Deletion of *A41L* from VACV WR does not alter immunity in the infected lung

Next, the infiltration of cells into the lungs and BAL fluids of mice infected intranasally with vA41L, vΔA41L or vA41L-rev was analysed by flow cytometry. No significant differences in the numbers of infiltrating macrophages, neutrophils, natural killer (NK) cells, $CD8^+$ or $CD4^+$ lymphocytes were found in the lung tissue or BAL fluids at days 3, 6 and 9 p.i. (data not shown). This was the case when either the absolute number of cells or the percentage of each cell type was compared.

The $CD8^+$ T-cell response in the lung was examined more closely by using ICS to determine the percentage of lung $CD8^+$ cells that produced IFN-γ in response to stimulation with the VACV-9 peptide (a newly identified H-$2D^d$-restricted epitope from VACV; D. C. Tscharke, unpublished results). In the gated lung-lymphocyte population, the percentage of $CD8^+$ cells that produced IFN-γ at either day 6 or 9 p.i. (Fig. 2a, b) was similar following infection with viruses that did or did not express A41. Likewise, after stimulation with VACV WR-infected P815 cells, no difference in the percentage (or absolute number) of IFN-γ-producing $CD8^+$ cells in the lung was detected on day 6 or 9 p.i. (data not shown).

To determine whether lung lymphocytes from mice infected with vΔA41L showed altered killing of VACV-infected autologous target cells compared with controls, chromium-release assays were performed. No significant difference in cytolytic activity was detected between groups on day 6 or 9 p.i. in the lungs (Fig. 2c, d).

Deletion of *A41L* increases $CD8^+$ T-cell responses in the spleen

In contrast to results with lung cells, ICS of splenocytes at days 6 and 9 p.i. revealed an increase in the percentage of $CD8^+$ cells that produced IFN-γ following intranasal infection with vΔA41L compared with infection with control viruses (Fig. 3a, b). This difference was significant on day 9 ($P<0{\cdot}05$). Similarly, when cells that produced IFN-γ in response to stimulation with VACV WR-infected cells were studied by ELISPOT, there was an increased number of IFN-γ-producing splenocytes (per 1 000 000 splenocytes) from vΔA41L-infected mice compared with control viruses (Fig. 3c, d) and this difference was significant ($P<0{\cdot}05$) on day 9 p.i. The numbers of spot-forming units for mock-stimulated splenocytes from all mice and stimulated splenocytes from mock-infected mice were negligible (data not shown).

Cytotoxic T-cell killing assays showed that splenocytes from mice immunized with vΔA41L lysed a significantly greater percentage of autologous VACV-infected target cells compared with control groups (Fig. 3e, f) ($P<0{\cdot}05$). Thus, although A41 had little effect on CTL activity at the primary

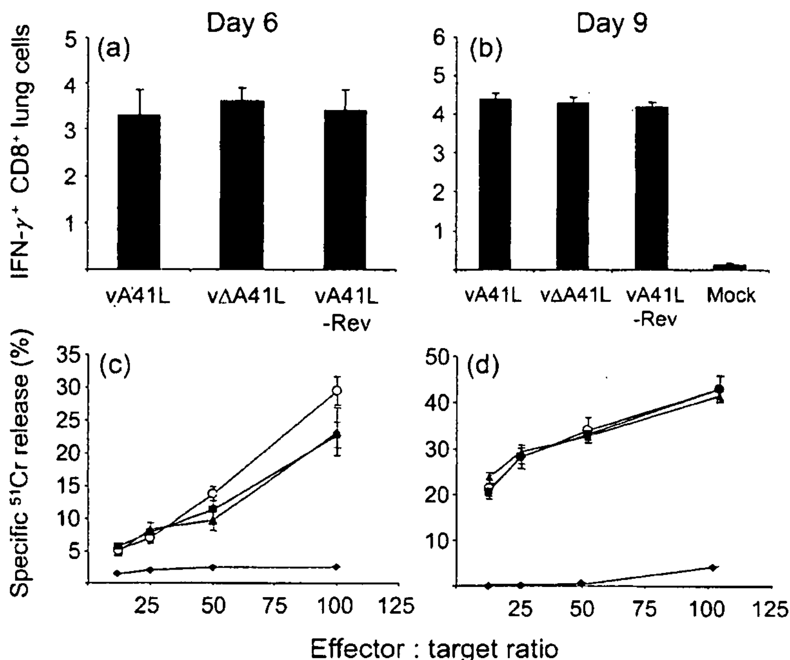

Fig. 2. $CD8^+$ T-cell response in the lungs of mice mock-infected (◆) or infected intranasally with VACV WR viruses [vA41L (▲), vΔA41L (○) or vA41L-rev (■)] as described in the legend to Fig. 1. At 6 (a, c) and 9 (b, d) days p.i., mice were sacrificed, lungs were excised and single-cell suspensions were prepared. (a, b) ICS analysis of lung cells revealed the percentage of $CD8^+$ T cells making IFN-γ after stimulation with P815 cells loaded with VACV-9 peptide. At days 6 (c) and 9 (d) p.i., the cytolytic activity of CTLs derived from lung was determined by ^{51}Cr-release assay. Cytolytic activity is presented as the mean percentage ± SEM.

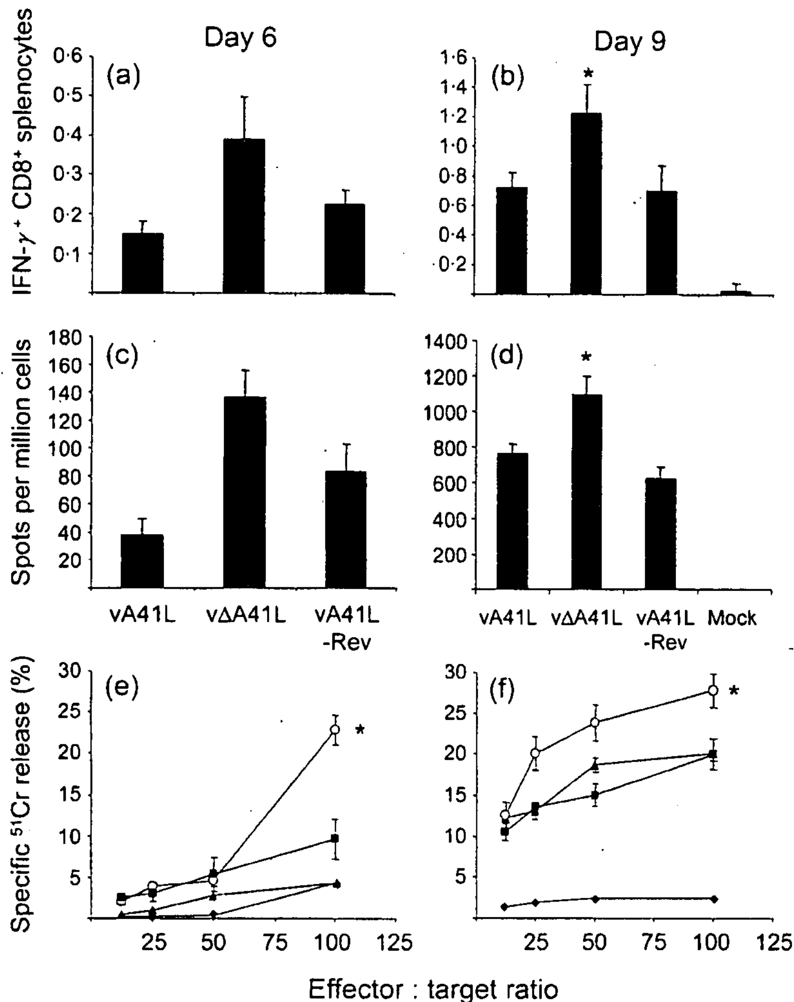

Fig. 3. CD8$^+$ T-cell response in the spleen of mice mock-infected (◆) or infected intranasally with VACV WR viruses [vA41L (▲), vΔA41L (○) or vA41L-rev (■)] as described in the legend to Fig. 1. At 6 (a, c, e) and 9 (b, d, f) days p.i., mice were sacrificed, spleens were excised and single-cell suspensions were prepared. (a, b) ICS analysis of lung cells revealed the percentage of CD8$^+$ T cells making IFN-γ after stimulation with P815 cells loaded with VACV-9 peptide. (c, d) ELISPOT analysis of IFN-γ-producing splenocytes at days 6 (c) and 9 (d) p.i. Splenocytes were stimulated with P815 cells that had been infected with VACV WR and the IFN-γ-producing cells were counted. At days 6 (e) and 9 (f) p.i., the cytolytic activity of CTLs derived from spleen was determined by ^{51}Cr-release assay. Cytolytic activity is presented as the mean percentage ± SEM. Significant differences (*$P<0{\cdot}05$) between vΔA41L and both wild-type VACV and vA41L-rev are indicated.

site of infection, the number of CTLs in the spleen, or their cytolytic effector function, was enhanced when A41 was absent.

Taken together, these data indicate that A41 interferes with the generation of optimal CD8$^+$ T-cell responses to VACV in the spleen.

Deletion of *A41L* from MVA enhances CD8$^+$ T-cell memory

VACV strain WR is not a vaccine strain. Therefore, to determine whether the enhanced immune response deriving from loss of the *A41L* gene from VACV WR was also true for vaccine strains of VACV, the *A41L* gene was deleted from VACV strain MVA and the immunogenicity was compared with wild-type and revertant controls. The parent virus selected was an MVA strain expressing the *P. berghei* circumsporozoite antigen (Schneider *et al.*, 1998), because this enabled the CD8$^+$ T-cell response to a foreign antigen to be evaluated in addition to the anti-VACV response. Mice were immunized subcutaneously with 10^8 p.f.u. parental (MVA-Pb), deletion (MVA-Pb-ΔA41L) or revertant (MVA-Pb-A41L-rev) virus on days 0 and 21. Mice were sacrificed on day 42 and the splenic CD8$^+$ memory immune response was assessed.

Splenocytes were stimulated with VACV WR-infected P815 cells (Fig. 4a) or *P. berghei* peptide (Schneider *et al.*, 1998) (Fig. 4b) and the number of IFN-γ-producing splenocytes was quantified by ELISPOT. Mice immunized with MVA-Pb-ΔA41L virus produced a significantly greater number ($P<0{\cdot}05$) of IFN-γ-producing cells than mice immunized with either control virus, irrespective of whether the splenocytes were stimulated with VACV-infected cells (Fig. 4a) or Pb9 peptide (Fig. 4b).

IFN-γ production by splenocytes was also examined by ICS. P815 cells that had been infected with VACV WR or pulsed with peptides (Pb9 or VACV-9) were used to stimulate splenocytes for 5 h before ICS. Analysis of gated lymphocytes revealed a significantly higher number ($P<0{\cdot}05$) of cells that produced IFN-γ in spleens of mice vaccinated with MVA-Pb-ΔA41L compared with control groups (Fig. 5). This increase was statistically significant for splenocytes stimulated with cells infected with VACV WR (Fig. 5a) or

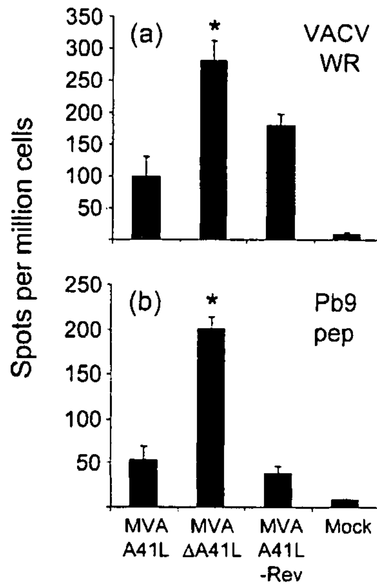

Fig. 4. ELISPOT analysis of IFN-γ-producing splenocytes after immunization with MVA. Groups of five BALB/c mice were mock-infected or infected subcutaneously with 10^8 p.f.u. of indicated viruses on days 0 and 21. Spleens were excised on day 42 and single-cell suspensions were analysed. Splenocytes were stimulated with P815 cells that had been infected with VACV WR (a) or incubated with Pb9 peptide (b) for 12 h. IFN-γ-producing cells were counted as described in the legend to Fig. 2. A significant difference between MVA-vΔA41L and both control viruses is indicated ($^*P<0{\cdot}05$). Data are presented as the mean ± SEM.

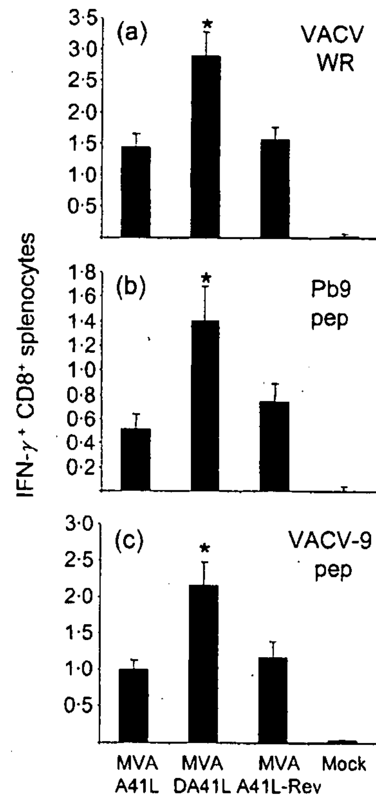

Fig. 5. IFN-γ-producing splenocytes after prime–boost immunization with MVA. Splenocytes were prepared as described in the legend to Fig. 4 and ICS was used to measure IFN-γ production after a 5 h stimulation with P815 cells infected with VACV WR (a) or loaded with peptide Pb9 (b) or VACV-9 (c). Data are the percentage of $CD8^+$ lymphocytes producing IFN-γ. Significant differences between MVA-vΔA41L and both MVA-A41L and MVA-vA41L-rev are shown ($^*P<0{\cdot}05$).

pulsed with the Pb9 peptide (Fig. 5b) or VACV-9 peptide (Fig. 5c).

ICS was also used to measure TNF-α production in the splenocytes of mice immunized with different recombinant MVA viruses (Fig. 6). Although the overall number of splenocytes producing TNF-α was lower than those producing IFN-γ, the number of cells producing TNF-α was higher following infection with MVA-Pb-ΔA41L than in control groups. This result was true when cells were stimulated with VACV-infected cells (Fig. 6a) or cells pulsed with Pb9 (Fig. 6b) or VACV-9 (Fig. 6c) peptides and, in each case, the difference was statistically significant. In conclusion, all of the methods used suggest that removal of A41 from MVA-Pb enhances the size of the antiviral $CD8^+$ T-cell memory pool.

Deletion of *A41L* from MVA improves vaccine efficacy

To examine whether deletion of *A41L* from MVA created a more effective vaccine, mice were vaccinated with 10^6 p.f.u. of each virus or were mock-infected and, 28 days later, were challenged with 3×10^6 p.f.u. VACV WR. This challenge dose represents approximately 300 LD_{50} for BALB/c mice of this age and, consequently, even vaccinated mice began to lose weight after infection (Fig. 7a). During the first 5 days p.i., a similar trend in weight loss was observed for the mice vaccinated with each virus. However, by day 6 or 7 p.i., animals infected with wild-type and revertant viruses had lost > 30 % of their original body weight and were sacrificed (humane end point). In contrast, mice vaccinated with MVA-Pb-ΔA41L started recovering weight by day 6 and survived the challenge (Fig. 7a). In addition, by day 7, mice vaccinated with MVA-Pb-ΔA41L showed significantly fewer signs of illness than mice immunized with wild-type and revertant controls and were starting to recover (Fig. 7b).

In another experiment, animals were vaccinated with a higher dose (10^7 p.f.u.), which induced better protection against the same challenge dose. At this immunization dose, all three groups of mice survived the challenge, but, as seen in the first experiment, animals immunized with the virus lacking A41 were protected better and had reduced weight loss (Fig. 7c) and signs of illness (Fig. 7d) than controls. Thus, vaccination with MVA-Pb-ΔA41L not only generated

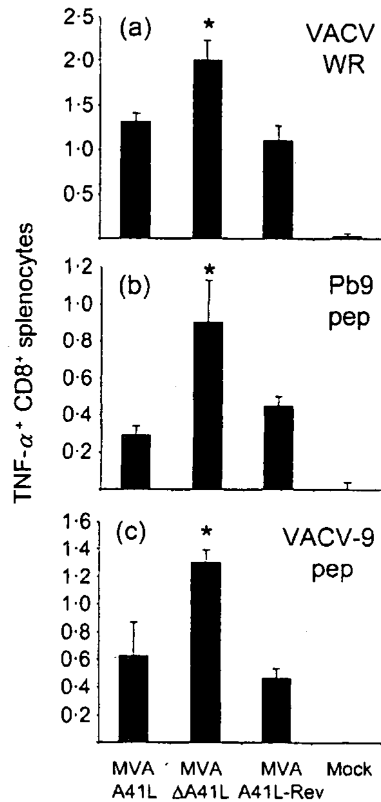

Fig. 6. TNF-α-producing splenocytes after prime–boost immunization with MVA. Experimental details were as described in the legend to Fig. 5 except that ICS was used to measure production of TNF-α rather than IFN-γ.

an enhanced memory CD8$^+$ T-cell response against VACV and foreign antigens, but it also conferred better protection against challenge with VACV WR.

DISCUSSION

Data are presented showing that deletion of gene *A41L* from VACV strains WR and MVA increased virus immunogenicity. VACV WR lacking A41 was slightly more virulent in an intranasal-infection model, but induced a stronger CD8$^+$ T-cell response in the spleen. The upregulation of VACV-specific CD8$^+$ T cells in the spleen was observed not only in the primary adaptive immune response to intranasal infection with VACV WR, but also in the memory response after subcutaneous immunization with MVA. Moreover, immunization with MVA lacking A41 induced better protection to challenge with a lethal dose of VACV WR. Collectively, these data show that deletion of *A41L* from VACV may result in a more immunogenic and efficacious vaccine, particularly if enhanced CD8$^+$ T-cell responses are important for vaccine efficacy.

The slight increase in virulence of vΔA41L in the intranasal model compared with control viruses was seen after infection with low virus doses only and not at the higher doses used previously (Ng *et al.*, 2001). This can be explained by higher doses of virus inducing a more severe infection and so masking the subtle difference induced by loss of gene *A41L*. Previously, it was observed that VACV WR strains lacking the IL-1βR (Alcamí & Smith, 1992, 1996) or CC chemokine-binding protein (Reading *et al.*, 2003a) were more virulent than controls expressing these proteins in this model. In the intradermal model, infection with vΔA41L caused a larger lesion size than controls, but the virus was cleared more rapidly than viruses expressing A41 (Ng *et al.*, 2001). In contrast, the slightly increased virulence in the intranasal model was not accompanied by an alteration in virus titres in the lungs or spleen. The altered virulence following intranasal infection might have been attributable to a difference in the cells infiltrating into the infected lung. However, analysis showed that removal of *A41L* had no significant influence on either the number of macrophages,

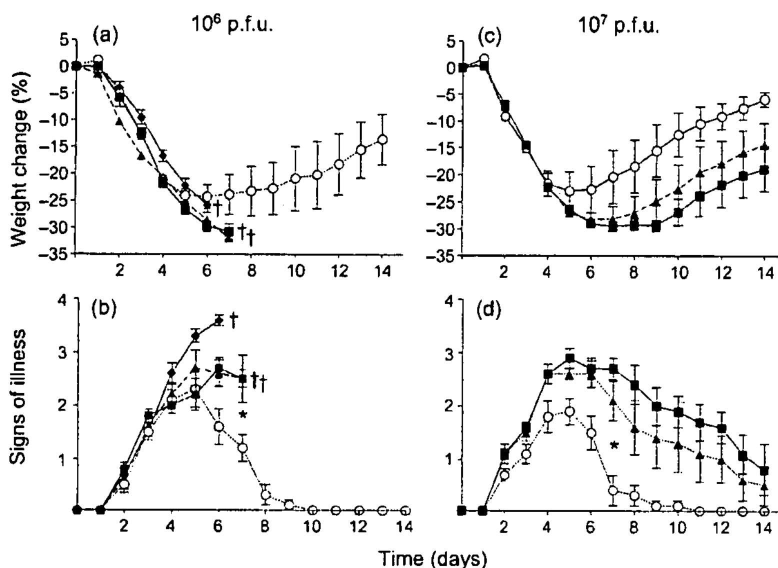

Fig. 7. Challenge of mice immunized with MVA viruses. Groups of five mice were mock-infected (◆) or immunized subcutaneously with 10^6 (a, b) or 10^7 (c, d) p.f.u. of the indicated viruses (■, vA41L; ○, vΔA41L; ▲, vA41L-rev) and challenged intranasally 28 days later with 3×10^6 p.f.u. VACV WR. Weight change (a, c) and signs of illness (b, d) were monitored daily and are presented as described in the legend to Fig. 1. Significant differences between vΔA41L and both control viruses are indicated (*$P<0.05$).

neutrophils, NK cells, CD8$^+$ and CD4$^+$ T cells migrating into the lung or their activation state (data not shown). So, if increased activation of cells or their infiltration into the lungs was the cause of enhanced virus virulence, these changes may have been in cells not analysed in our study.

Investigations into the CD8$^+$ T-cell response at days 6 and 9 p.i. by using three methods provided strong evidence that A41 influences the adaptive immune response. Firstly, the percentage of CD8$^+$ T cells in the spleen that produced IFN-γ in response to VACV-infected cells or a VACV epitope in ICS assays was increased after infection with vΔA41L. This trend was seen early in the adaptive immune response (day 6) and then, more profoundly, at the height of the adaptive response (day 9). Surprisingly, statistically significant upregulation of the IFN-γ response was not observed for the CD8$^+$ T lymphocytes infiltrating into the lung. Secondly, the ICS data were supported by the results of ELISPOT assays. Finally, increased CTL activity, the hallmark of activated antiviral CD8$^+$ T cells, was also seen in the spleens, but not lungs, of mice infected with vΔA41L. Reduction of CD8$^+$ T-cell numbers or their functionality in the spleen, but not in the lung, suggests that A41 may be inhibiting the migration of cells to the spleen (e.g. antigen-presenting cells or T cells). However, it is also possible that A41 interferes at a more indirect level early in the immune response, resulting in poorer activation of CD8$^+$ T cells in the spleen.

A possible role for A41 in subverting cellular migration is also suggested by its sequence similarity to known poxvirus chemokine-binding proteins. However, only weak binding to the CXC chemokines IP-10 (CXCL10), ITAC and MIG (CXCL9) was identified and A41 did not inhibit the chemotactic activity of these CXC chemokines in biological assays. Interestingly, IP-10 and MIG are active as chemotactic factors for stimulated, but not for resting, T cells and have been shown to have a role in lymphocyte migration to the lung (Loetscher *et al.*, 1996). Additionally, VACV expressing either IP-10 (crg-2) or MIG downregulated IFN-γ production and NK cytolytic activity in the spleen in mouse infections (Mahalingam *et al.*, 1999).

Several immunomodulatory proteins expressed by VACV have an inhibitory influence on the host cellular immunity. A 3β-hydroxysteroid dehydrogenase encoded by gene *A44L* inhibits the percentage of IFN-γ-producing CD8$^+$ cells in the lung and the cytolytic activity after intranasal infection of mice (Reading *et al.*, 2003b). This enzyme synthesizes steroid hormones, such as glucocorticoids, that have a general immunosuppressive effect. Similarly, the IL-18-binding protein (gene *C12L*) expressed by VACV WR targets the Th1 response (Symons *et al.*, 2002; Reading & Smith, 2003). In mice infected intranasally with a virus lacking *C12L*, there was an enhanced CTL activity in the lung and spleen and a dramatic increase in the percentage of IFN-γ-producing CD8$^+$ T cells in the lung. In neither case was the memory CD8$^+$ T-cell response to VACV measured. Here, we show that removal of *A41L* from MVA enhances the antiviral CD8$^+$ T-cell memory response, as has also been shown for the VACV IL-1βR (Staib *et al.*, 2005).

Lastly, the efficacy of MVA lacking *A41L* as a vaccine was tested by immunizing mice with MVA with or without *A41L* and subsequently challenging the animals with a lethal dose of VACV WR. Viruses lacking *A41L* induced better protection than controls, demonstrating a biological relevance that extends beyond the T-cell assays. Whilst we have shown that CD8$^+$ T-cell responses are increased when A41L is deleted, we cannot assume that the increased protection against a VACV challenge is due only to CD8$^+$ T cells. Measurement of the antibody titres after infection with VACV WR (or MVA) with or without A41 by ELISA (using extracts of VACV-infected cells and purified recombinant B5 protein) and intracellular mature virus neutralization assay showed no significant differences between the groups (data not shown). However, it remains possible that other parameters might contribute to the enhanced protection.

In summary, this study has revealed a novel property of A41 immunobiology and demonstrated that the immunogenicity of VACV-based vaccines can be increased. The influence of A41 on the CD8$^+$ splenocytes gives insight into the immunomodulatory role of this secreted protein and its deletion illustrates the potential benefit of removing other immunomodulators from the MVA genome to improve vaccine potency.

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